Biosynthesis of Biotin in Microorganisms

VI. Further Evidence for Desthiobiotin as a Precursor in *Escherichia coli*

CHIK H. PAI AND HERMAN C. LICHSTEIN

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada, and College of Medicine, University of Cincinnati, Cincinnati, Ohio 45219

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Biotin auxotrophs were isolated from *Escherichia coli* K-12. One of the mutants was unable to grow on desthiobiotin and accumulated a large amount of a vitamer in medium when growing on an optimal concentration of biotin. The production of the vitamer was inhibited in the presence of an excess amount of biotin. The vitamer was identified as desthiobiotin on the basis of biological activities, avidin combinability, and chromatographic characteristics. The mutant lacked the ability to convert desthiobiotin to biotin. These results further support the hypothesis that desthiobiotin is a normal intermediate in the biosynthesis of biotin in *E. coli*.

Desthiobiotin has been suggested as a precursor of biotin in a variety of microorganisms (2, 6, 8, 9, 12, 16). However, there has been no direct evidence to suggest that the vitamer is a normal intermediate of the biotin pathway. Indeed, a pathway to biotin has been proposed without the intermediate function of desthiobiotin (7).

In our previous report (13), desthiobiotin was shown to be converted to biotin by a resting-cell system of *Escherichia coli*. The level of the conversion system was controlled by biotin; that is, the system was repressed and derepressed depending on the availability of biotin to the organism during growth. These findings suggested that desthiobiotin is a normal intermediate of biotin.

To study the biosynthetic pathway of biotin in *E. coli*, a number of biotin mutants were isolated and characterized. One of the mutants provided an opportunity to examine further the role of desthiobiotin in the biosynthesis of biotin.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 served as the parent strain from which the following biotin mutants were isolated: K-12-22, K-12-11, and K-22-20. *E. coli* strain 58 was kindly provided by E. L. Tatum.

Growth media and conditions for growth. A glucose-salts medium (10) supplemented with 4 g (per liter) of Vitamin Free Casamino Acids (Difco) was employed as a basal medium. The Casamino Acids were purified by charcoal adsorption to remove traces of biotin (14). For growth of the biotin mutants, the basal medium was supplemented with biotin or desthiobiotin. Biotin and desthiobiotin solutions were sterilized by filtration and added aseptically to the basal medium. All cultures were incubated at 37°C with vigorous shaking. Growth was measured tur-

bidimetrically in a Klett-Summerson photoelectric colorimeter fitted with a red filter (no. 66); 1 Klett unit = 0.00133 mg (dry weight) of *E. coli* per ml.

Microbiological assays. Biotin was assayed with *Lactobacillus plantarum* (ATCC 8014) by the method of Wright and Skeggs (18), and with *Saccharomyces cerevisiae* (ATCC 9896) according to the procedure of Hertz (5). Slight modifications in both methods were used as described elsewhere (10). *L. plantarum* responds only to biotin and biotin sulfoxide of the naturally occurring forms; *S. cerevisiae* responds to a variety of biotin vitamers including desthiobiotin.

For the determination of avidin combinability, known amounts of vitamers and avidin (Worthington Biochemical Corp., Freehold, N.J.) were mixed aseptically and allowed to react at room temperature for 30 min. A sample was then assayed for residual biotin activity by use of the yeast.

Bioautography of biotin vitamers. The method used is described elsewhere (12). A small amount (0.01 ml) of culture filtrate was spotted directly on a filter paper strip without its being concentrated. The 7,8-diamino-pelargonic acid, 7-keto-8-amino-pelargonic acid, and 7-amino-8-keto-pelargonic acid were kindly provided by I. Kumashiro.

Isolation of biotin mutants. *E. coli* K-12 was grown and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) according to the method of Adelberg, Mandel, and Chen (1). The treated cells were incubated in the basal medium supplemented with 1×10^{-3} µg/ml of biotin for 3 hr. The culture was diluted properly, and 0.1 ml was spread on basal agar medium supplemented with a suboptimal concentration of biotin (1×10^{-4} µg/ml). The plates were incubated at 37°C for 24 hr, and small colonies were picked with sterile tooth picks and examined for biotin requirement. The replica-plating technique was used also.

Assay of the biotin-synthesizing system. The amount of biotin synthesized from desthiobiotin by a resting-

cell system was measured. Preparation of resting-cell suspensions, description of the reaction mixture, and conditions for assay were as described previously (13). The activity was expressed in terms of micromoles of biotin synthesized per milligram (dry weight) of cells per hour. The system was repressed when grown in the presence of 5×10^{-3} $\mu\text{g/ml}$ of biotin; it was derepressed when the excess amount of biotin was removed suddenly by avidin, which combines with biotin thus making it unavailable to the cells (13). For derepressed cell preparations, cultures were harvested at 0.5 generation time (25 min) after the addition of avidin.

RESULTS

Growth responses of a number of biotin mutants are shown in Table 1. Strain K-12-20 was the only biotin mutant of *E. coli* unable to grow on desthiobiotin. All strains of biotin mutants that have been reported grow on desthiobiotin just as well as on biotin (4, 15).

To determine if biotin precursors were accumulated during growth, the wild type (K-12) and the mutant strain (K-12-20) of *E. coli* were grown in the basal medium with or without supplementation of graded amounts of biotin, and the culture filtrates were assayed for biotin by use of *L. plantarum* and *S. cerevisiae* (Table 2). For the wild type, 280×10^{-4} μg (per ml) of vitamers was accumulated with very little biotin appearing. In the culture filtrate of the mutant, practically all of the added biotin was used up, and large amounts of vitamers were accumulated. With an optimal concentration of biotin (5×10^{-4} $\mu\text{g/ml}$), the amount of the vitamers accumulated was about eight times greater than in the culture filtrate of the wild type. It is also of note that the production of the vitamers was inhibited when an excess amount of biotin was added to the medium. Since the biosynthesis of biotin is controlled by biotin via repression (12), the vitamers could

be the precursors of the biotin that the mutant was unable to convert to biotin.

The nature of the vitamers was examined by bioautography (Fig. 1). A single peak was revealed from the culture filtrates of the wild type and mutant strains. The chromatographic properties of the vitamer were compared with those of other known biotin vitamers (Table 3). The unknown vitamer exhibited R_F values in three solvent systems compatible with desthiobiotin. The vitamers were inactive for *L. plantarum* but were active for *S. cerevisiae* and *E. coli* strain 58. The unknown vitamer was inactivated completely

TABLE 2. Accumulation of biotin vitamers

Strain	Biotin in basal medium ($\mu\text{g/ml}$)	Growth ^a	Biotin equivalent (10^{-4} $\mu\text{g/ml}$) as assayed by	
			<i>Lactobacillus plantarum</i>	<i>Saccharomyces cerevisiae</i>
K-12	None	290	0.1	280
K-12-20	None	40	0.1	320
	1×10^{-5}	52	0.1	450
	5×10^{-5}	95	0.1	900
	1×10^{-4}	160	0.3	1,300
	5×10^{-4}	275	0.8	2,100
	1×10^{-3}	270	1.8	900
	1×10^{-2}	280	2.0	50

^a Klett readings after 18 hr of incubation. With the no. 66 filter, 1 Klett unit = 0.00133 mg (dry weight) of *Escherichia coli* per ml.

TABLE 1. Biotin requirements of mutant strains of *Escherichia coli*

Strain	Additions to basal medium		
	None	Biotin ^a	Desthiobiotin ^a
58	5 ^b	330	345
K-12-22	7	340	330
K-12-11	6	335	340
K-12-20	3	330	5

^a Biotin = 5×10^{-4} $\mu\text{g/ml}$; DL-desthiobiotin = 10^{-3} $\mu\text{g/ml}$.

^b Klett readings after 24 hr of incubation; media and growth conditions are as described under Materials and Methods. With the no. 66 filter, 1 Klett unit = 0.00133 mg (dry weight) of *E. coli* per ml.

FIG. 1. Bioautography of culture filtrate of *E. coli* K-12 and K-12-20. The solvent system used was *n*-butanol and water (43:7). The wild type was grown in the basal medium and the mutant in the basal medium supplemented with 5×10^{-4} $\mu\text{g/ml}$ of biotin. The culture filtrates were obtained after 18 hr of incubation. One Klett unit = 0.00334 mg (dry weight) per ml of *Saccharomyces cerevisiae*.

TABLE 3. *Chromatographic properties of the unknown biotin vitamer accumulated by strain K-12-20 and authentic known vitamers*

Vitamin	R _F		
	n-Butanol-acetic acid-water (4:1:5)	n-Butanol-water (43:7)	t-Butanol-methylethyl ketone-water-ammonium hydroxide (4:3:2:1)
Unknown	0.87	0.65	0.71
Desthiobiotin	0.88	0.63	0.70
Biotin	0.80	0.36	0.66
7-Amino-8-ketopelargonic acid	0.66	0.23	
7-Keto-8-aminopelargonic acid	0.72	0.31	
7,8-Diaminopelargonic acid	0.37	0.06	

when a slightly excess amount of avidin was added in assay tubes. This suggests the presence of an intact urea ring in its structure. These results indicate the identity of the unknown vitamer to be desthiobiotin.

The ability of the mutant strain K-12-20 to carry out the conversion of desthiobiotin to biotin was examined (Table 4). Three strains of *E. coli* were grown under conditions in which the biotin-synthesizing system was repressed or derepressed (13). Unlike the wild type (K-12) and the mutant strain 58, which can be grown on desthiobiotin, no activity could be demonstrated in the mutant strain K-12-20 under either condition.

DISCUSSION

The role of desthiobiotin as a precursor of biotin had been suggested on the basis of the following observations: (i) it replaces biotin as a growth factor in a number of microorganisms (8); (ii) it is converted to a substance having the properties of biotin by a number of microorganisms (6, 12, 16); (iii) it is found in culture filtrates of some wild-type microorganisms (2, 9).

However, the possibility remained that desthiobiotin was simply an active precursor but not a normal, obligatory intermediate in the biosynthesis of biotin (17).

Use of auxotrophic mutants to define the biotin pathway was attempted by Tatum (15), who reported that a mutant of *Penicillium chrysogenum*, which was unable to grow on desthiobiotin, accumulated a substance having biotin-like activity for a number of microorganisms including another biotin mutant of the mold. The vitamer was considered to be desthiobiotin solely on the basis

TABLE 4. *Biosynthesis of biotin from desthiobiotin by Escherichia coli**

Strain	Biotin synthesized per mg (dry weight) of cells per hr	
	Repressed	Derepressed
	μmoles	μmoles
K-12	<1	46
58	7	104
K-12-20	<1	<1

* The reaction tubes contained: potassium phosphate buffer (10^{-2} M, pH 6.8); magnesium sulfate (2.84×10^{-3} M); glucose (4.4×10^{-2} M); ($\pm$)-desthiobiotin (10^{-6} M); cells (0.19 to 0.38 mg, dry weight, per ml); and water to make a final volume of 5.0 ml. The reaction tubes were allowed to equilibrate at 37 C for 10 min before addition of bacterial suspensions. Incubation was carried out at 37 C for 60 min, after which the bacteria were removed from the reaction mixture by centrifugation. The supernatant solution and the cells were assayed for (+)-biotin by use of *Lactobacillus plantarum*. Control tubes containing no desthiobiotin were included in the enzyme assay, and all experimental values were corrected for the endogenous values.

of its biological activity, and no further study was made regarding its identity. However, recent studies by Eisenberg and Maseda (3), using the same mutant strain of *P. chrysogenum*, revealed that the predominant vitamer produced by the mutant was not desthiobiotin but rather an unknown biotin vitamer previously described by Eisenberg (2) and Ogata et al. (9).

In the present studies we made a similar approach, using biotin auxotrophs of *E. coli*. It is evident from the results presented that a loss of the ability to convert desthiobiotin to biotin was accompanied by a loss of the ability to synthesize biotin and by an increased accumulation of desthiobiotin during growth. This suggests that desthiobiotin is a normal, obligatory intermediate in the biosynthesis of biotin in *E. coli*. This hypothesis was strengthened further by the finding that the accumulation of desthiobiotin was completely inhibited by an excess amount of biotin. This is in agreement with our previous report that the production of desthiobiotin, as well as its conversion to biotin, was under physiological control via repression by biotin (12, 13).

Similarities among microorganisms regarding production and utilization of desthiobiotin suggest that the role of desthiobiotin may be the same for most, if not all, organisms. The only microorganism reported not to produce desthiobiotin

in its growth medium is a strain of *Achromobacter* studied by Lezius et al. (7).

A complete understanding of reactions leading to the synthesis of biotin from desthiobiotin awaits isolation and characterization of the enzyme or enzymes involved.

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